Identification of Mucin2 as a strong promoter for gut-specific genes

Research Thesis for the completion of Undergraduate Research Distinction

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Abstract

As per capita consumption of poultry continues to increase, it is necessary for the poultry industry to optimize production to accommodate the growing demand. Discovery of gut-specific genes could significantly improve poultry production through a better understanding of genes that relate to nutrition, feed efficiency, and the microbiome. In the present study, the Mucin 2 promoter is used to drive overexpression of green florescent protein (GFP) in intestinal tissue. Through comparison of gene expression in different tissues, the gut specificity of Mucin 2 expression was confirmed in mice and humans. RT-PCR data indicates that poultry have Mucin 2 expression exclusively in their small and large intestines, suggesting the Mucin 2 promoter can be used to drive expression of target genes in the gut. Lentiviral particles were generated to contain the 2.9kb Mucin 2 promoter and GFP gene downstream of the promoter. The vector was then transfected in a human intestinal epithelial cell line (Caco-2 cells) and confirmed to exhibit green fluorescence in these gut cells. A microneedle and injector was used to inject the lentiviral particles, containing the target gene constructs, into the subgerminal cavity of the blastoderm. Seventeen chimeric founder lines were generated after injection of 105 wild type quail eggs. Chimeric hatchlings were grown to sexual maturity and mated with wild type quail to determine which chimeras had the transgene present in their gametes. Their offspring are currently being genotyped by PCR to detect vector integration. Currently, one transgenic offspring has been confirmed. Transgenic birds will be maintained as generation 1 (G1) lines. The offspring of G1 lines will be analyzed by Western blot and immunofluorescence imaging to confirm GFP expression only in gut epithelial cells. Following confirmation of gut-specificity, the promoter can be utilized to drive overexpression of target genes in intestinal tissue that could improve production and reduce the impact of the poultry industry on the environment.

Introduction

Some important components of the avian digestive tract are the small intestine, large intestine, and ceca. These visceral organs have functions including water and nutrient absorption as well as fermentation. The identification of gut-specific genes and promoters that could modify the function of these tissues could be very beneficial in improving poultry production and efficiency. Mucins are a family of gel forming glycoproteins that can be divided into two groups: secreted and membrane-associated Mucins (Voynow and Rubin, 2009). Mucin2 (Muc2) codes for a gel-forming protein that aids in the protection of the gastrointestinal tract (Rousseau *et al*., 2004). Any disruption or complication with Muc2 could potentially lead to reduced performance by the animal (Kermanshahi *et at*., 2015). Microarray data as well as PCR analysis, using broiler chicken tissue distributions, indicated that Muc2 is specific to intestinal tissue. Currently transgenic quail are being generated to confirm Mucin 2 as a gut-specific gene. Transgenic quail will express an enhanced green florescent protein (eGFP) gene in intestinal tissue under the control of the Mucin 2 promoter. This novel study could have a significant impact on the poultry industry by providing information to increase food safety and reduce environmental impact.

Materials and Methods

Animal Use

Animal care and use procedures were approved by Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. Japanese quail (*Coturnix coturnix japonica*) were housed at The Ohio State University Poultry Facility in Columbus, Ohio. A standard starter or breeder diet and water was provided to the animals *ad libitum*. Sacrificed animals were ethnized via CO_2 inhalation.

Tissue Collection

Tissue distributions were taken from seven week of broiler chickens. Fat, thigh muscle, pectoralis muscle, heart, liver, lung, kidney, spleen, duodenum, jejunum, ilium, cecum, and large intestine tissues were collected. These tissues were collected in order to determine the intestinal specificity of Muc2.

Total RNA Extraction and Polymerase Chain Reaction

Total RNA from the chicken tissues was isolated using Trizol reagent (Invitrogen) according to manufacturer's protocol. RNA quality was assessed by gel electrophoresis, and quantity was measured by a Nanodrop spectrophotometer (Thermo Fisher Scientific). cDNA was generated by using 1 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Invitrogen) with conditions, 65 °C for 5 min, 37 °C for 52 min, and 70 °C for 15 min. cDNA samples were used to conduct qPCR for Muc2 using 2 primers; Muc2/F, 5'TGACTGAATGTGAAGGAACATGTG3' and Muc2/R, 5'TTCATTTTGATGTTAAGCTGATGG3'. To get a linear amplification of PCR products, mucin 2 cDNA was amplified for 32 cycles with denaturation at 95°C for 25 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s. Equal volumes of each tissue were loaded into the agarose gel to determine the gut specificity of Mucin2. RPS13 was used as a control.

Construction of Lentiviral Vector and Virus Particles

The 2.9 kb sequence in the 5' upstream region of *cMucin2* gene was amplified from chicken genomic DNA by PCR with a forward primer containing ClaI site, 5' – AATCGATTTT AGCAGCAGAGAATCCCCA – 3', and a reverse primer containing PacI site, 5' - AGTTAATTAAGGCTAAGGTGGGTGAACTGTGA, and was then cloned into pCR2.1-TOPO

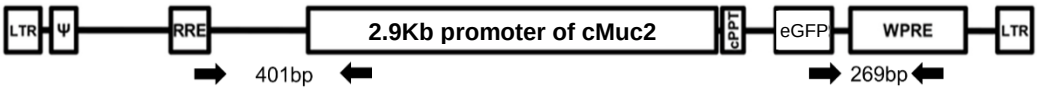

Figure 1. Schematic of Mucin2 lentiviral vector

vector (Invitrogen, Carlsbad, CA). Two restriction enzymes, ClaI and PacI were used to digest the pCR2.1 recombinant vector. This produced the 2.9 promoter fragment. The 2.9 kb promoter replaced a RSV promoter that had been constructed previously (Shin *et al*., 2008). The resulting vector, pLT-cMuc2-eGFP, was designed to express the *eGFP* gene specifically in the intestinal tissue and be driven by the Muc2 promoter. Co-precipitation of calcium phosphate and the vector was used to produce lentiviral particles. 24 hours prior to the transfection, 293FT cells were plated on 100mm culture dishes in complete medium. This medium consisted of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Inc., Grand Island, NY), with 10% fetal bovine serum (FBS; Life Technologies Inc.), 1% penicillin/streptomycin (pen/strep; Life Technologies Inc.), 1 mM MEM sodium pyruvate (Life Technologies Inc.), ans 0.1 mM MEM non-essential amino acids (Life Technologies Inc.). 9 µg of pLT-Mucin2-eGFP, 9 µg of ViraPower Packaging Mix (Life Technologies Inc.), and 87 µl of 2M calcium solution (Clontech Laboratories Inc., Mountain View, CA) were added to a final volume of 700 µl of Sterile H2O (Clontech Laboratories Inc., Mountain View, CA) to prepare the transfection solution. 700 µl of 2× HEPES-Buffered Saline (HBS) (Clontech Laboratories Inc.) were then added dropwise while slowly vortexing the solution. The transfection solution was incubated for 5 minutes at room temperature, then added dropwise to the complete medium. Following 10 hours of transfection, 5ml of fresh complete medium was used to replenish the medium. After 48 hours the supernatant was collected then filtered through a 0.22µm pore sized filter. The titer of lentiviral supernatants was measured by a standard ELISA method using the Lenti-X p24 Rapid Titer Kit (Clontech

Laboratories Inc.) after the non-concentrated viral supernatants were serially diluted. The supernatant was pelleted via centrifugation at 25,000 rpm for two hours with an ultracentrifuge (L7-65R, Beckman Coulter, Fullerton, CA), resuspended in Opti-MEM as a 100× concentrated lentiviral particle, and stored as 40 µl aliquots at –80°C until time of use. The lentiviral vector was used to infect CaCo2 cells, a human intestinal cell line, to ensure the vector was working properly (Sambuy *et al*., 2005).

Microinjection of stage X embryos

105 stage X fertilized quail eggs were injected with virus particle through microinjection. The quail eggs were cleaned with 70% ethanol. The eggs were placed on one side for approximately 4 hours at room temperature. This positioned the embryos to the side of the egg. 2-3µl of the lentiviral particle was injected into the subgerminal cavity of the blastoderm of fertilized egg via a small, 5mm, window on the side of the egg. The window was made with fine tipped tweezers.

Hatching and Mating of Chimeric Founders

Of the 105 embryos microinjected with the lentiviral particle 18 hatched (17.1%) following a 17 day incubation period. 17 of these chimeric founders survived to sexual maturity at 7 weeks of age. Currently, 142 G1 birds have been screened. Mature birds were mated to wildtype quail to produce 17 Generation 0 (G0) founder lines. G1 birds are still being screened in order to identify more positive G1 animals.

Detection of Transgenic Quail by PCR

At two weeks of age feather pulp was collected from G1 offspring and a DNA extraction was performed. Genotyping PCR was later performed to determine integration of the transgene. After reaching sexual maturity, the transgenic G1 bird was mated to a wildtype quail and the G2

generation was produced. G2 offspring are currently being screened to test for the integration of the transgene.

Western Blot Analysis

Western blot was performed to confirm the positive identification of transgenic birds and to confirm that the lentiviral vector was properly working. The procedures followed was the same as that of a previous study (Li *et al*., 2012). Following SDS-PAGE and transfer to polyvinylidene fluoride (PVDF) membrane, the membrane was incubated overnight at 4°C with the anti GFP primary antibody (Sigma Aldrich St. Louis, Mo) at a 1:3000 dilution in 1x Tris-buffered saline containing 0.05% tween-20 (TBST) with 4% non-fat dry milk. After washing in 1x TBST, Western blots were incubated with Rabbit IgG HRP-conjugated secondary antibody (R&D systems Inc., Minneapolis, MN) at room temperature for 1 hour. The membrane was then washed with 1x TBST before the addition of Amersham ECL plus Western Blotting Detection Reagents (GE Healthcare Biosciences, Pittsburgh, PA), and the blots were then exposed to Hyperfilm (GE Healthcare Biosciences) to visualize the target proteins.

Results

PCR analysis indicated that Muc2 was gut-specific. Further study will indicate if Muc2 is specific to the mucosa of the intestines.

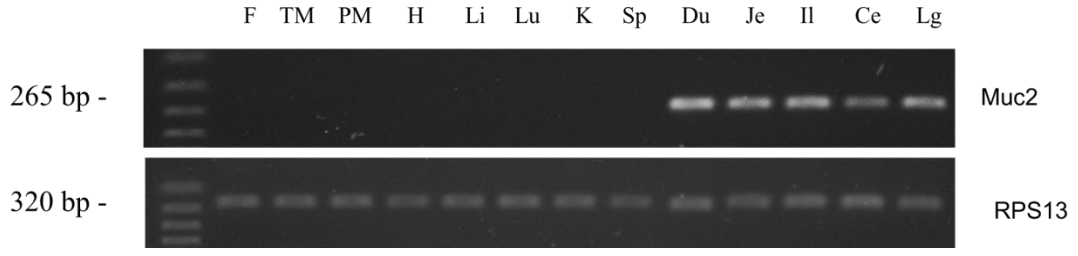

Figure 2. PCR results for tissue distribution of 7-wk- old broilers

Florescent microscopy indicated that the lentiviral vector successfully infected Caco2 intestinal cells, as evidenced by the detection of GFP. The control cells that were plated did not demonstrate florescence. However, the cells that had been infected with 20ul of the virus did demonstrate florescence. Western blot indicated that the virus was dose-dependent. At 27kDa a thicker band was present for 20ul of the virus as opposed to 10ul of the virus.

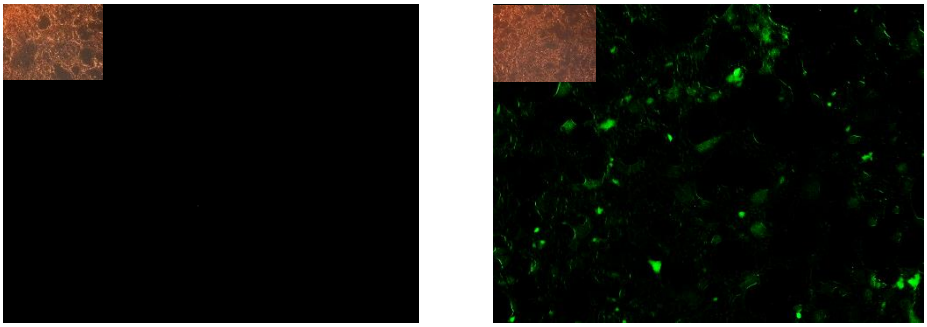

Figure 3. Left: control cells under fluorescence. Right: Caco2 cells infected with 20ul of virus under fluorescence.

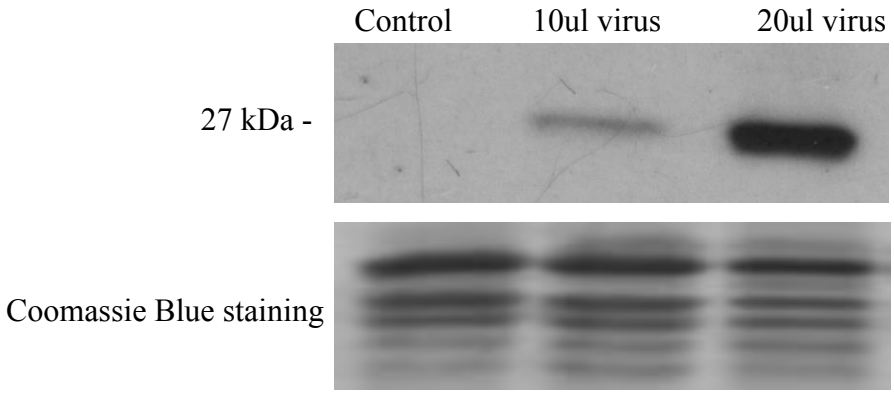

Figure 4. Western blot (12% gel) of Caco1 intestinal cell infection to detect GFP

Genotyping PCR has revealed one transgenic G1 progeny for further study. Currently, six G2 birds have been screened at the embryo stage. Two of the animals were positively identified as transgenic via genotyping PCR.

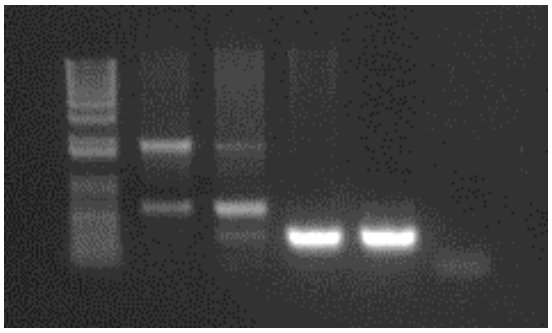

Figure 5. G1 genotyping on 1% agarose gel. **Lane 1:** 1 kb ladder **Lane 2:** Wild type **Lane 3:** Wild type **Lane 4:** Transgenic bird **Lane 5**: (+) **Lane 6:** (-)

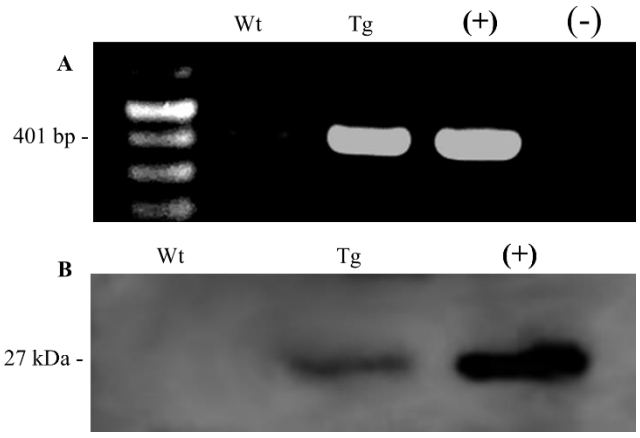

Figure 6. **(A)** G2 genotyping on 1% Agarose gel. **(B)** Western blot for detection of GFP protein.

Discussion

PCR and infection of Caco-2 cells were used to determine the gut specificity of Mucin2. This novel study could potentially provide the poultry industry with a method for studying gut specific genes in the future. Following the generation of additional chimeric founder lines the intestinal-specificity of Mucin2 will be further confirmed, and identify Muc2 as a novel gut-specific gene.

Japanese quail were used in this study because quail have been identified as a good model for genetic studies. Quail have a short generation time, lay a large number of eggs, and have a short

incubation period. They have also been used in other genetics studies previously (Ahn *et al*., 2015).

Future research will include generating more chimeric lines to produce more G2 progeny. Those progeny will be either be used in the functional study or be kept as breeders. Tissue distributions will be taken and tested under ultraviolet light. This will determine the tissue specificity of *eGFP* expression. Due to the gut specificity of Mucin to it can be hypothesized that only the intestinal tissue will fluoresce because it is under control of the Muc2 promoter. Following the conformation of the tissue under ultraviolet light immunohistochemistry will be used to detect the presence of GFP in the intestinal tissue. Further study will also be conducted to determine if Mucin2 is specific to either the epithelial or smooth muscle of the intestine. Once multiple chimeric lines are generated the expression patterns in each line will be compared to determine if all of the lines have the same expression patterns. Different stages of development will also be tested to determine when the transgene is expressed.

In conclusion, this study further confirmed the gut specificity of Mucin2. The intestinal expression of *GFP* will be confirmed following the generation of more progeny. Eventually this construct could, be useful to the industry by overexpressing genes other than *GFP* in the intestine. These overexpressed genes could potentially increase animal production or decrease the environmental effect of the poultry industry, and overall improve efficiency to meet the growing demand.

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